REMARKS

Courtesies extended to Applicants' representative in the personal interview held on October 29, 1999, and the telephone interview held on August 3, 2000, are acknowledged with appreciation.

The present invention is based on the discovery that recombinase-encoding nucleic acid constructs can be incorporated *into the genome* of embryonic stem cells so as to be expressed at high levels in the germ line by means of a germ-line specific promoter, without expression to a functionally significant extent in either ES cells (i.e., in culture) or in embryonic or adult somatic tissues.

Accordingly, the present invention provides transgenic mammalian embryonic stem (ES) cells whose genomes are transformed with recombinase-encoding nucleic acid constructs (claims 12-15, 18-24, and 26) useful for preparation of transgenic animals, such as mice. These nucleic acids comprise a mammalian germ-line promoter operatively linked to a recombinase encoding gene. The genome of invention ES cells can further contain a transcriptionally active selectable marker flanked by recombinase recombination sites and/or a nucleic acid fragment flanked by recombinase recombination sites specific for a different recombinase than the recombination sites that flank the selectable marker. Because the germ-line promoter directs recombination events in the germ-line, but only to a de minimus amount in other tissues, embryos can be derived from such ES cells that contain a transgenic allele, such as is caused by homologous recombination at recombinase target site(s).

In addition, when the ES cells contain a nucleic acid fragment flanked by recombinase recombination sites specific for a different recombinase than the recombinase expressed in the germ line (i.e., different than the recombination sites that

flank the selectable marker), such as under control of an inducible or tissue specific promoter, recombination of the target site can be controlled to occur in a specific (i.e. somatic) tissue or in an inducible manner. In addition, because the ES cells contain a germ-line specific promoter operatively linked to the recombinase encoding gene, the transcriptionally active selectable marker can be excised by passage of the genome derived from said embryonic stem cells through gametogenesis. ES cells obtained by crossing the genome of the transgenic gamete with a wild type genome can be used to obtain ES cells in which the transgene is stably incorporated, into the genome, but the selectable marker is excised. Excision of the marker without excision of the allele of interest allows any phenotype that is observed to be more confidently ascribed to the mutation of interest rather than to some combination of that mutation and the transcriptionally active marker.

In accordance with the invention there are also provided methods for the production of transgenic animals (i.e., containing recombinant alleles), as well as methods for conditional assembly of functional genes for expression in eukaryotic cells by recombination of individual inactive gene segments from one or more genes of interest using the invention ES cells containing recombinase responsive nucleic acids.

Claims 1-10, 12-16, 18-26 and 28-45 were pending before this communication. By the present communication, claims 1-10, 16, 25, 33, and 45 are canceled without prejudice (claims 3 and 45 being withdrawn as subject to a restriction requirement imposed herein by the Examiner), claims 12-14, 18-24, 26, 28, 32, 35, 40, 43, and 44 are amended, and new claims 46-51 are added to define Applicants' invention with greater particularity. Therefore, claims 12-15, 18-24, 26, 28-32, 34-44 and 46-51 are currently pending. These amendments and new claims add no new matter as the amended and new claim language is fully supported by the specification and original claims.

The Rejection Under 35 USC § 112, First Paragraph

Applicants respectfully traverse the rejection of claims 1-10 (now canceled), 12-16, 18-26 and 28-44 for alleged lack of enablement under 35 USC § 112, First Paragraph. Applicants disagree with the Examiner's assertion that the Specification fails to enable use of a germ-line specific promoter (Office Action, pages 3-4.)

With regard to the tissue specificity of germ-line promoters employed in the practice of Applicants' invention, Applicants submit that it is well known in the art that the term "tissue-specific promoter" encompasses promoters that produce certain low levels of activity in tissue other than the target tissue (In fact, Applicants define the term "tissue specific" as referring to "the substantially exclusive initiation of transcription in the tissue from which a particular promoter, which drives expression of a given gene is derived" Specification, page 8, lines 15-18). Therefore the Examiner's assertion that the specification fails to enable use of a "germ line-specific" promoter is not consistent with the art-understood meaning of the term.

Applicants respectfully disagree with the Examiner's assertion that the Specification allegedly fails to enable one of skill to determine whether the expression of any gene is functionally significant in the instant invention (Office Action, page 3). Applicants teach that "functionally significant" expression in tissue does not occur when expression is 100 times less than in testes. As previously noted, use of the "germ-line specific promoter" resulted in expression of the transgene in the heart, brain and spleen in all transgenic individuals tested at a level "more than 100 fold lower than that observed in testis" (Specification, page , line 24). The Specification teaches that such a low level is considered to "not show any ectopic activity" (Specification, page 17, lines 30-32; emphasis added). Thus, according to Applicants, "functionally significant" expression must be at a level greater than 1/100 of the level of expression in testes.

Applicants further disagree with the Examiner's assertion that the embodiment of the invention transgenic animal illustrated in the Specification (i.e., a transgenic mouse that expresses a marker gene) is allegedly "not of use" because the mouse does not have a phenotype. Invention first generation transgenic animals having a transgenic allele (i.e. animals derived from an invention embryonic stem cell) all have the phenotype that the transgenic allele is produced by operation of the recombinase upon passage of the genome derived from the embryonic stem cell through gametogenesis. Animals derived from invention stem cells without passage of the genome of the stem cells through gametogenesis have the phenotype that the recombinase transgene is incorporated into a chromosome.

In addition, Applicants respectfully disagree with the Examiner's assertion that "embodiments encompassing ES cells used to make mice expressing marker genes are not enabled because such cells have no disclosed use" (Office Action, page 3). The utility of invention transgenic animals and transgenic ES cells would be readily understood by those of skill in the art as research tools useful for studying the effect of knocking out any gene targeted by the invention recombinase recombination mechanism. Applicants respectfully submit that the Specification fully illustrates utility of the invention ES cells at the least as a research tool for conveniently producing transgenic animals, such as mice, that are functionally wild-type but can contain heterologous nucleic acid constructs in their genomes passed by germ-line transmission. It is up to the researcher to determine what phenotype is to be studied using the invention ES cells and non-human animals prepared using the ES cells.

The Examiner appears to be taking the position that, because an invention is useful to researchers, it is unpatentable. However, the utility of the presently claimed ES cells and the transgenic rodent produced using them are in a currently available form and a "real world value" can be placed on them. The commercial market for such ES cells and transgenic animals provides clear evidence of such value. Thus, Applicants respectfully submit that the invention ES cells and transgenic animals themselves have a known function and are valuable reagents for

researchers. What gene will be targeted by the recombinase recombination sites is the choice of the researcher who will use Applicants' invention as a research tool.

In addition, Applicants respectfully disagree with the Examiner's assertion that enablement is allegedly not provided for methods of producing a lethal allele using the invention constructs and methods because the level of chimerism required to allow survival and transmission of a transgene is not taught in the specification and because the specification does not teach how to "mask" lethality by cross-breeding with a wild-type animal. (Office Action, pages 4-5). Applicants respectfully submit that those of skill in the art would understand that the "masking" of lethality happens automatically in a certain percentage of the animals obtained. Thus, no teaching in the Specification regarding the "level of chimerism" or "how to 'mask' lethality" is required for those of skill in the art to practice the embodiment of the invention wherein a lethal allele is obtained.

In addition, Applicants respectfully submit that animals of commercial purpose can be produced without undue experimentation by those of skill in the art using invention methods and constructs. The function and reliability of the recombinase recombination system for disrupting a target gene was well understood in the art at the filing date of the present application, as is shown by the cited art and as acknowledged by the Examiner in the Office Action (see Office Action, page 10). The Examiner has provided no reasons to support the position that the recombinase recombination system in transgenic animals derived as illustrated by Applicants using the mouse model will not work if the animal is not a mouse.

Therefore, in view of the above amendments and remarks, Applicants submit that all pending claims are fully enabled by the teachings of the Specification. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

The Rejection Under 35 U.S.C. § 112, Second Paragraph

The rejection of claims 33, and 40-42 under 35 U.S.C. § 112, Second Paragraph, for alleged indefiniteness is respectfully traversed.

With regard to claim 33, Applicants respectfully disagree with the Examiner's assertion that the term "essential" lacks definition in the specification and in the art, resulting in alleged indefiniteness. Moreover, it is respectfully submitted that the phrase "an essential portion of a gene of interest" as used in claim 33 is not indefinite because those of skill in the art would readily understand that an "essential" portion of DNA is a portion of the sequence that is required to produce a biological function of the DNA in the cell, whether the DNA encodes a functional protein or transcription regulatory regions, or whether the DNA is part of an intron whose function cannot be determined. Stated another way, those of skill in the art would understand that deletion of a portion of DNA that is not "essential" would not interfere with the native biological function of the DNA or its protein product in the cell or organism. Furthermore, Applicants submit that those of skill in the art using Applicants' invention as a research tool would know how to determine when an "essential" portion of DNA has been excised.

However, to expedite prosecution and reduce the issues, by the present communication, claim 33 is canceled, thus removing the grounds of the rejection for alleged indefiniteness as to claim 33.

With regard to claim 40, Applicants respectfully disagree with the Examiner's assertion that the phrase "recombinase responsive construct" is indefinite. However, the issue was rendered moot by Applicants' deletion of the phrase from claim 40 in the previous Response mailed herein on June 14, 1999.

With regard to claim 44, Applicants respectfully disagree with the Examiner's assertion that the claim is unclear because it "does not result in a step wherein the recombinant livestock (with a non-wild type phenotype) occurs" (Office Action, page 4). A phenotype in transgenic animals created using the method of claim 44 occurs simply by carrying out the required steps of the method. However, to expedite prosecution and reduce the issues, by the present communication claim 44 is amended to add a phrase explicitly requiring that a recombinant non-human animal is produced "by operation of the recombinase upon passage of the genome derived from the embryonic stem cell through gametogenesis." In view of this amendment, Applicants respectfully submit that amended claim 44 is definite because it expressly requires production of the recombinant livestock animal as well as requiring a distinct phenotype of the recombinant animal.

In view of the above amendments and remarks, Applicants respectfully submit that amended claims 33, 40 and 44 are definite under 35 U.S.C. §112, Second Paragraph.

The Rejection Under 35 U.S.C. § 103

I. Applicants respectfully traverse the rejection of claims 1-2, 4-5, 10, 12-16 18-19 24-26 and 28-44 for alleged obviousness over Gu et al. (Science :265-1-3-106, 1994, hereinafter "Gu") in view of Zambrowicz *et al.* (*Biology of Reproduction 50*:65-72, 1994, hereinafter "Zambrowicz"), and Lakso et al. (Proc. Natl. Acad. Sci, 93:5860-5865, 1996). Claims 1-10 are canceled by the present communication without prejudice, thus obviating the rejection as to the subject matter of those claims.

Applicants invention transgenic stem cells, as defined by amended claim 12, distinguish over the teachings of the cited art, whether taken alone or in combination, by requiring mammalian embryonic stem cells *whose genomes contain* a nucleic acid construct comprising a germ-line-specific promoter operatively associated with a

recombinase coding sequence, and wherein the recombinase is not expressed in the embryonic stem cells in culture.

Applicants' invention transgenic stem cells, as defined by amended claim 13, further distinguish over the cited art by requiring that *the genome of the embryonic stem cells contain* two nucleic acid constructs, one with a transcriptionally active marker gene under the control of a germ-line specific promoter flanked by recombinase recombination sites, and one having a polynucleotide target flanked by recombinase recombination sites. If the nucleic acid fragment is a transcriptionally active selectable marker, such embryonic stem cells can be isolated using the marker.

Applicants' methods for excision of the selectable marker from the embryonic stem cells, as required by amended claim 28, and for production of transgenic non-human transgenic animals, as required, for example by amended claims 32, 38 and 44, further distinguish over the cited art by requiring passaging the genome derived from such transgenic embryonic stem cells through gametogenesis will produce a transgenic non-human animal having an allele, for example by deletion of the marker gene in the germ-line, as required by claim 40. For example, Applicants have shown that in a rodent system an efficiency of as high as 92% recombination of the target allele can be achieved using this method "Of 112 target alleles transmitted by males of all 5 lines, 103 or 92%, were recombined." (Gorman, page 14604, Col. 2, top).

Applicants further teach that unless the marker gene and its transcriptional sequences are excised in the germ-line of the transgenic offspring, any phenotype detected in the offspring is not confidently ascribed to the presence of the allele because in some cases an interfering effect has resulted from the gene encoding the marker.

Applicants' invention methods further distinguish over the combined disclosures of the references by requiring production of offspring from invention ES cells that contain recombination sites that are specific to two different recombinases, such as Cre and FLP. One can be used to

trigger deletion of the marker during gametogenesis, and the other can be used later to remove the flanked target DNA to create a different transgenic allele in the offspring. Applicants have shown that loxP-flanked targets remain and are not recombined in the somatic tissues of mice that contain ProCre transgenes, but that more than 90% of the progeny sired by these males inherit a Cre-recombined target.

By contrast, Gu fails to disclose mouse ES cells *whose genome contains* a nucleic acid construct comprising a germ-line-specific promoter operatively associated with a recombinase coding sequence, wherein the recombinase is not expressed in the embryonic stem cells in culture. Instead, Gu teach that a Cre expression plasmid, in which expression is driven by a broadly expressed promoter, can be inserted into stem cells that contain a target DNA sequence previously inserted into the chromosome of a naïve ES cell line. The Cre expression plasmid is not integrated into the chromosome (in fact chromosomal integration would be counterproductive in Gu's approach) and the recombinase Cre is expressed in the ES cells. The transient expression of Cre *from an extrachromosomal plasmid sequence* in the ES cells themselves (partially) recombines the target. However, recombination of the target results in loss of the extrachromosomal Cre plasmid. Thus, a mouse obtained from Gu's transgenic ES cells, for example by injection into a blastocyst, *would not contain the recombinase transgene in its genome*.

Therefore, in Gu's method one strain of transgenic mice is produced in which Cre is expressed and then this first strain of transgenic mice is bred to a different strain of transgenic mice that contain a target gene. In progeny produced from crossbreeding these two strains of transgenic mice, the target is excised in a tissue specific manner. However, Gu's method for production of a transgenic mouse requires creation of and mating between two sexually mature transgenic mouse strains, one in which a Cre transgene is expressed in a cell-type specific manner (in T-cells) and a second one in which a transgene flanked by two loxP recombination sites is carried. There is no suggestion in Gu of how to use embryonic stem cells to accomplish the same goal without raising two sexually mature animals. In addition, presumably the desired

null allele would be found in no more than about 40% of the offspring of such a mating using the method of Gu (page 105, third column, bottom). The Gu method thus requires much additional time and expertise because two different transgenic mouse strains are required. Based on this analysis, Applicants submit that the present invention provides a substantial improvement over Gu's methods.

In addition, Gu suggests no advantage to be obtained from substantially germ-line specific expression of a recombinase since Gu is completely silent regarding use of a germ-line specific promoter operatively associated with a recombinase gene so that the recombinase gene is expressed only in germ-line tissue. Thus, Gu fails to provide motivation for creation of a transformed gamete from which ES cells can be obtained wherein the transcriptionally active selectable marker is excised and/or for further creation of a transgenic allele in a non-human animal using such ES cells.

Zambrowicz does not overcome all of these deficiencies in the disclosure of the primary reference. Zambrowicz discloses that testis nuclear proteins bind only to a specific region of the testis-specific Prm-1 promoter so as to regulate spermatid-specific transcription and, more specifically, "so as to recognize sequences within and immediately adjacent to a CRE-like sequence" (page 70, Col. 1 bottom, to Col. 2 top). However, Zambrowicz fails to disclose or suggest placing the testis-specific promoter into ES cells for any purpose, much less that a germ-line promoter operatively linked to a recombinase transgene could be integrated into the genome of ES cells in such a way that recombinase would not be expressed in the ES cells (i.e., in culture). Thus, like Gu, Zambrowicz is silent regarding methods for using the combination of any germ-line promoter in a nucleic acid construct containing a gene that encodes Cre, or any other germ-line promoter in a nucleic acid construct, to obtain insertion or deletion of a target gene into embryonic stem cells.

Moreover, Applicants respectfully submit that Zambrowicz fails to disclose or suggest Applicants' method, as defined, for example, by claims 35 or 40, for obtaining an offspring from

a mammalian embryonic stem cell wherein the marker gene has been deleted so that the mouse appears to be perfectly wild-type until recombination is triggered via an inducible or tissue specific promoter in an additional nucleic acid construct having recombinase recombination sites flanking a target nucleic acid fragment.

Therefore, Applicants respectfully submit that the combined disclosures of the references fail to teach or suggest creation of ES cells that contain *a chromosomally integrated copy* of a recombinase transgene that is not expressed in the ES cells, but would be expressed in the germ-line of derived chimeras at usefully high levels. In fact, the literature as a whole teaches that many genes whose expression is highly tissue-specific in the intact animal are unexpectedly expressed in ES cells in culture. In addition, the cited art fails to teach or suggest that a recombinase transgene can be introduced *into the chromosomes* of embryonic stem cells prior to (or even simultaneously with) introduction of the target sequence into the chromosome of the same cells. This feature of the present invention effects a great savings of labor and/or time over any methods suggested by the combined disclosures of the cited art.

Moreover, based on the foregoing analysis of the cited references, it is respectfully submitted that the Examiner has erroneously relied upon hindsight reconstruction of the references in light of Applicants' teachings in formulating the rejection for alleged obviousness. It is well settled in patent law that the Examiner is not allowed to selectively pick and choose elements or concepts from the various references so as to arrive at the claimed invention using the claims as a guide. Hindsight is not a proper criteria for resolving the issue of obviousness.

In view of the above amendments and remarks, Applicants respectfully submit that *prima facie* obviousness of present claims 12-15, 18-19, 24-26, 28-44 and 46-51 is not established by the Gu-Zambrowicz-Lakso combination of references. Accordingly, reconsideration and withdrawal of this rejection are respectfully requested.

II. Applicants respectfully traverse the rejection of claims 1, 6-9 and 20-23 for alleged obviousness over Gu in view of Zambrowicz and Lakso as applied in Section I above and further in view of Onouchi *et al.* (*Mol. Gen. Genet* 247:653-660, 1995, hereinafter "Onouchi") Office Action, page 10). The rejection is moot with respect to claims 1 and 6-9, which are canceled without prejudice.

The discussion presented above with respect to claims 20-23 concerning the insufficiency of the Gu-Zambrowicz-Lakso combination of references for disclosing or suggesting the invention nucleic acid constructs and their use in methods of making transgenic stem cells and/or transgenic animals derived from such stem cells having a transgenic allele is equally applicable here.

Applicants respectfully submit that Onouchi fails to overcome the shortcomings of the Gu-Zambrowicz-Lakso combination of references because Onouchi is absolutely silent regarding transgenic embryonic stems cells *whose genome contains* any type of germ-line-specific promoter operatively associated with any type of recombinase coding sequence. Thus, Applicants respectfully submit that the Gu-Zambrowicz-Lakso-Onouchi combination of references also fails to suggest invention embryonic stem cells, as defined by claims 20-23. Accordingly, reconsideration and withdrawal of this rejection are respectfully requested.

Conclusion

In view of the above amendments and remarks, reconsideration and favorable action on claims 12-15, 18-24, 26, 28-32, 34-44 and 46-51 are respectfully requested. In the event any

matters remain to be resolved in view of this communication, the Examiner is encouraged to call the undersigned so that a prompt disposition of this application can be achieved.

Respectfully submitted,

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